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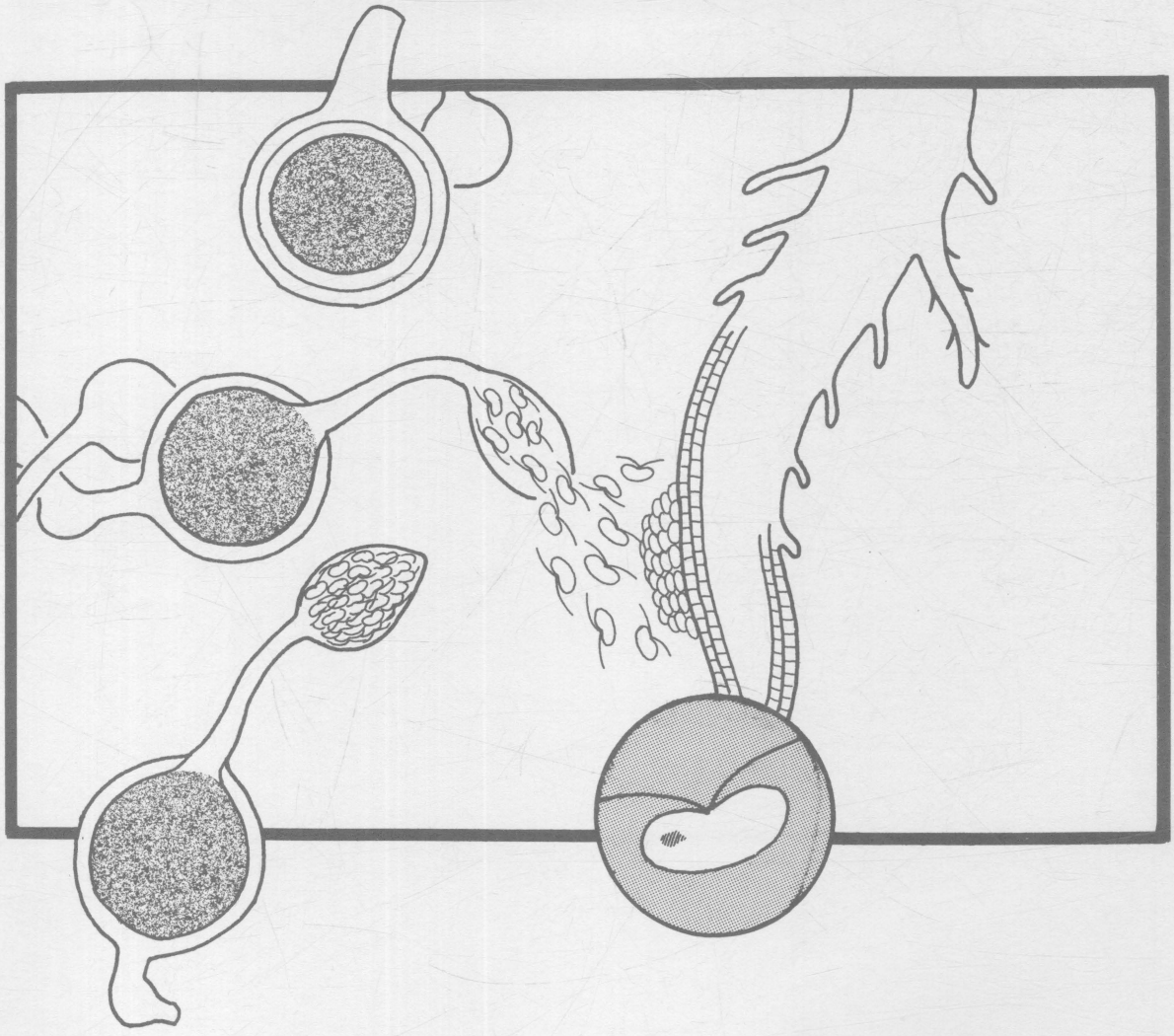
Useful Methods for Studying *Phytophthora* in the Laboratory



THE OHIO STATE UNIVERSITY
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Contents

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This is a compilation of methods, some old and some new, that have been found useful in Ohio for isolation, study and identification of *Phytophthora* spp. It is not a review of all methodology used for investigation of *Phytophthora*. The methods have been developed primarily for *P. sojae,* but have proven useful for such species as *P. cactorum, P. capsici, P. cinnamomi, P. citricola, P. citrophthora, P. cryptogea, P. erythroseptica, P. medicaginis* and *P. megasperma.* Some of these methods have been discussed previously (17).

I. Detection and Isolation of *Phytophthora*

A. Staining structures in diseased tissues.

Sporangia can be frequently seen on the surface and oospores within rotted tissues if they are teased apart. Clearing and staining rotted tissue is helpful. Autoclave or boil diseased tissues for 10 min in a chloral hydrate-acid fuchsin clearing-straining solution (7). Then place tissues in lactophenol. To observe structures, squash cleared and stained tissues between two slides. Remove the upper slide and mount in lactophenol. *Phytophthora* and *Pythium* oospores and sporangia appear deep pink. Nematodes, nematode eggs and endotrophic mycorrhizal fungal structures also will appear pink. Plant tissues will be tinged pink. Caution: Avoid breathing chloral hydrate fumes. Trypan blue also may be used, in which case the fungal structures will stain blue.

B. Inducing sporulation on rotted tissues.

Heterothallic *Phytophthora* spp. do not produce oospores in rotted tissues. If sporangia are not present on the root surface they can be induced to form as follows. Place bits of well-washed rotted and partially rotted tissues in 10 ml distilled water. Incubate at room temperature (24 C) for 24-48 hr. Sporangia of most species will be formed. Incubation at 18 C will induce sporulation in low-temperature species. Replace water with fresh water and chill for 15 min for good zoospore formation. Add two drops of lactophenol-trypan blue solution to terminate development and preserve structures.

C. Detection with monoclonal antibodies.

1) *Detection in diseased tissues.* Agri-Diagnostics Associates, 2611 Branch Pike, Cinnaminson, NJ 08077, has developed a monoclonal antibody kit for detection of *Phytophthora* in diseased tissues. This kit is now manufactured by Neogen, Inc.(620 Lesher Pl, Lansing, MI 48912) and is available through Sigma Chemical Co (P.O. Box 14508, St. Louis, MO 63178-9916). The procedure is simple and requires about 10 min to run an assay. *Phytophthora* antigen is extracted from diseased tissues by rubbing and grinding with an abrasive pad. The abrasive pad is placed in extraction solution and shaken 20 times. A filter top is placed on the container and 6 drops of the extraction solution are forced through the filter and placed at the intersection of 3 holes in a detector unit. The detector unit has a membrane with bound *Phytophthora* antibody under one hole, and positive and negative controls under the other holes. The membranes cover an absorptive column that soaks up the liquid passing through the holes. After the extract drops have passed through the holes, three drops of detector solution (antibody conjugated with peroxidase) are added and, as soon as the liquid is absorbed, wash-solution is added, then a peroxidase color reagent, and then a wash-solution. Each stage takes 1-2 min for the liquid to absorb. After the fifth step, the detectors are ready to read. No color is a negative reaction, and intense blue a strong reaction, indicating high amounts of *Phytophthora.* The amount of *Phytophthora* is roughly correlated with the intensity of the color reaction. The antibodies used will react strongly with all

Phytophthora spp., very weakly with *Pythium* spp., but not with other common pathogens. A multiwell, double sandwich ELISA kit, which is cheaper and more convenient for multiple sampling, also is available using the same technology.

2) *Detection and quantitation in soil:* Agri-Diagnostics Associates (see address above) has developed an immunoassay for quantitation of *Phytophthora* in soil. The procedure involves extraction of oospores from soil, grinding the oospores, and performing an ELISA test on the oospore homogenate. Fifty grams of the soil sample are placed in a 125-ml polypropylene bottle, and 75-ml of water is added. The soil is suspended in the water by shaking vigorously for 20 sec. Water is added to fill the bottle and a thick, plastic cover slip (capture slip) is placed on top so that its undersurface is in contact with the liquid without air pockets or loss of liquid. After 30 min, the capture slip is removed, and the oospores and debris adhering to it are scraped into a dentist's amalgamator capsule containing a steel ball. The capture slip is washed with 50μl of water that is added to the capsule. The oospores are ground by vibrating the capsule for 30 sec at high speed in the amalgamator. Then 100μl of clarifying solution is added to the homogenate, which is moved to a centrifuge tube containing 1.5 ml of antigen extraction buffer. The capsule halves are placed in the tube, open ends down, the mixture is vortexed, then centrifuged 5 min at 2500 rpm. The supernatant is filtered through a Porex filter and an ELISA test performed with a *Phytophthora* multiwell kit. *Phytophthora* levels are estimated by comparing with 200, 100, 35 and 0 laboratory-prepared oospore standards.

D. Isolation of *Phytophthora.*

1) *Conventional media:* Wash tissues thoroughly to remove any soil or loose plant debris. Flushing tissues with 70% ethanol for 5 sec, then flushing off the ethanol with sterile distilled water, is the simplest and most effective procedure for surface sterilization. Soaking tissues briefly in 1:4 chlorox (10 sec for thin tissues, 30 sec for thicker tissues, or 1 min for large tissues) can be used but is less effective and more damaging to *Phytophthora*. Cut small wedges from advancing margin of rot or edge of a lesion. Place wedges on lima bean or dilute V-8 juice agar. **Invert the agar medium over the tissue** to reduce competition from bacteria. Potato dextrose agar is a very poor medium. Lima bean and dilute V-8 juice agar are satisfactory for most species. For *P. infestans,* lima bean agar prepared with the extract of 150 g/l frozen lima beans is best.

2) *Selective media:* Isolation of *Phytophthora* is easier and more consistent using selective media. Washing, surface sterilization and tissue selection should be the same as discussed above. Dilute V-8 juice agar is a good medium for use with selective inhibitors. One standard selective medium, PVP (22), contains pimaricin and PCNB for inhibition of fungi, with vancomycin for inhibition of bacteria. A second useful inhibitor combination, PBNIC (15), has Benlate, PCNB and Iprodione for inhibition of fungi, with neomycin sulfate and chloramphenicol for inhibition of bacteria. A third combination, PARP (11), has pimaricin and PCNB for fungal inhibition and ampicillin and rifampicin for bacterial inhibition.

All three media are excellent for isolation. Pimaricin combinations are more selective, but cannot be autoclaved or stored in the light. All ingredients in PBNIC can be autoclaved except Iprodione. None of the media completely eliminate all contaminating fungi and bacteria. None of the media completely inhibit Mucoraceae (i.e., *Rhizopus, Mucor, Mortierella,* etc.). For best results, selective agar should be inverted over diseased tissue sections and transfers made from the fungus that grows through the agar. Many other selective inhibitor combinations have been devised. See the review by Tsao (20) for more information.

E. Isolation of *Phytophthora* in the presence of *Pythium.*

Pythium spp. are invariably present on both healthy and diseased roots, crowns and lower stems. Most *Pythium* species will grow more rapidly than *Phytophthora* on traditional media. Consequently, *Phytophthora* is very difficult to isolate from root tissues. Several procedures can be tried to selectively isolate *Phytophthora.*

1) *Hymexazol (Tachigaren) in the medium:* Hymexazol at rates of 0.02 and 0.05 g/liter will inhibit many *Pythium* spp. (21) but not others such as *P. irregulare* or *P. vexans* and can be added to selective media. Some slow-growing *Phytophthora* spp. such as *P. sojae* are inhibited by hymexazol, even at the low rate. If hymexazol doesn't work some other procedures can be tried.

2) *Tissue selection: Pythium* generally is confined to the roots or to badly rotted lower stems. *Pythium* can be avoided by selection of active stem lesions as high as possible above the soil. Also, *Pythium* generally is confined to the outer cortex. Generally, *Pythium* can be killed in outer layers with surface sterilization. If only root rot is present, care should be taken to isolate

from the center of the rotted tap root, since *Pythium* generally is confined to the cortical tissues. Split the stem, and pull the root apart by extending the stem split. Aseptically remove small pieces of rotted tissue from the center of the exposed root surface.

3) *Induce and isolate zoospores:* Place the thoroughly washed infected tissues in sterile distilled water. Monitor frequently after 4 hrs. Zoospores will be present in less than 24 hr, if they are going to form. As soon as zoospores are produced, capture them with a Pasteur pipette. Place 0.2 ml of zoospore suspension on the surface of a medium such as dilute lima bean or V-8 juice, spread the suspension with a hockey stick (0.5 cm × 25 cm glass rod with 6 cm at one end bent at a 45^0 angle) and invert the agar in the plate. The zoospores will germinate and the mycelium will grow to the agar surface. Be advised that some selective media are inhibitory to zoospore germination. Generally, *Pythium* will not form zoospores as rapidly as *Phytophthora* when diseased tissues are placed in water.

F. Isolation of *Phytophthora* from soil.

1) *Direct isolation on selective media: Phytophthora* is difficult to isolate from soil because *Pythium* grows on the same selective media and will outgrow *Phytophthora*. Direct plating of soil on selective media has been successful for a number of *Phytophthora* spp., but only by experts who can distinguish young *Phytophthora* from *Pythium* colonies by mycelial characteristics. See the list compiled by Tsao (20). A typical method for isolation from soil is that of Papavizas *et al.* (16) for *P. capsici.*

2) *Apple baiting:* Baiting methods are still the simplest and most effective way to isolate *Phytophthora* from soil. Campbell's apple fruit method (3) is still being used. He filled a hole in apple fruit (yellow apples are best) with soil, incubated for 1 week and isolated from the edge of the rotted area surrounding the hole. *Phytophthora* causes a firm rot. Soft rot is indicative of other fungi. Tsao (20) has listed many other baiting techniques. Several additional baiting methods have been selected for consideration because of their simplicity and wide applicability.

3) *Pear baiting:* Green pears have been used to bait *Phytophthora* from water (8). Wash pears to remove residual waxes and fungicides. Suspend pears in the water for 3 days. Remove from water and incubate for 3 days in a moist chamber. Isolate from the lesions that develop. This method is not selective for *Phytophthora* but may be useful for detection of this fungus in water or flooded soil. *Pythium* and various of the Mucorales also will cause lesions on pears suspended in water.

4) *Lupine baiting:* The lupine method (5) consists of placing soil in the bottom of a container (32 oz Dart polystyrene cups), flooding with water, and suspending roots of 4-day-old lupine seedlings in the water (Dart sundae cups with holes in bottom nested in 32-oz cup to support seedlings). *Phytophthora* will form lesions on the tap root from which it can be isolated. This method is suitable for other large seedlings such as soybeans.

5) *Alfalfa seedling baiting:* The alfalfa seedling method (13) consists of floating germinated seedlings in water over soil in a petri dish. The seedlings are wounded before being placed in the water. Lesions formed can be used for isolating *Phytophthora*, or placed in water to induce sporulation and thus demonstrate the presence of *Phytophthora.* This method is adaptable to all small seedlings.

6) *Soybean seedling bioassay:* For large-seeded plants such as soybean, the following bioassay is useful for detecting *Phytophthora* in soil and for obtaining isolates for identification and race typing. Residual inoculum of *P. sojae* in soil probably consists of oospores. Thus, planting seed in a soil may not detect *Phytophthora* since oospores require some time to germinate and seedlings may be large enough to escape damage by the time enough active fungus is present for infection. Therefore, soil that has overwintered or dried out should be treated to induce oospore germination before planting a bioassay. Flood the soil for 1 hour, then drain and air dry until the moisture content approaches approximately −300 mb matrix potential (soil cracks or pulls away from side of container although it is still damp). Containers of soil should then be placed in polyethylene bags to halt further drying and incubated at room temperature for a total of 2 weeks from flooding. Oospores germinate and form sporangia during this period.

Then plant seed of a highly susceptible cultivar, 15 seed/10 cm dia container, water just enough for good germination or cover with wet vermiculite, and place into plastic bag to prevent drying out during germination. Three days after planting, when the seedling roots are 5 cm long, flood the containers for 24 hours. Drain off the flood water and incubate in the greenhouse for 10 days. During this period seedlings emerge and damp-off. *Phytophthora* can readily be isolated from collapsed hypocotyls. For vegetable crops such as pepper, 1-month-old seedlings can be transplanted into the soil after the sporangia induction period (2 weeks at −300 mb) and containers flooded in

two weeks after seedlings become established. If *P. capsici* is present, good stem lesions form from which the fungus can readily be isolated.

7) *Leaf disc baiting:* A particularly useful tissue bioassay is the soybean leaf disc method (4). Moisten soil to −0.3 bar matric potential and hold in a sealed polyethylene bag at 25 C for 2 wk for sporangial production. Place a representative sample (5-20 g moist wt) in a small beaker and flood with distilled water to a depth of 1-2 cm above the soil. Avoid mixing soil with water. Remove surface film and floating debris by wiping the surface of the water with several pieces of tissue paper. Place freshly punched leaf discs (0.5 cm dia) from the unifoliolate leaves of 10- to 15-day-old soybean seedlings (highly susceptible cultivar such as Sloan) on the surface of the water, 20 to 30 leaf discs per beaker. Two hours after flooding the soil, remove the leaf discs from the water, blot for a few seconds on sterile paper towels, and place on a *Phytophthora*-selective medium. Incubate plated leaf discs in the dark at 25 C. Mycelium of *Phytophthora* can be readily distinguished growing beyond the margins of the water-soaked (infected) leaf discs in 3 days. This method is selective for *Phytophthora* because of the brief period the leaf disc is in contact with the water. *Phytophthora* produces zoospores from preformed sporangia quicker than *Pythium.*

II. Purification and Maintenance of Cultures

A. Separation from contaminants.

Contaminant-free cultures are a necessity for both storage and sporulation of *Phytophthora.* Most cultures isolated from diseased tissue or soil are contaminated with bacteria, and many with fungi. The best method of separation of *Phytophthora* from contaminants is to place a piece of culture on a plate of selective medium and **invert the entire disc of agar medium in the plate** (18). Then cut the edges of the inverted agar so that the surface seals to the bottom of the plate. The inoculum thus is trapped in a bubble underneath the agar. Bacteria will not grow through agar. Fungal contaminant growth is inhibited by the selective medium. As soon as the *Phytophthora* mycelium has grown beyond the bubble, take a thin piece of the culture from the surface to establish a clean culture. Sometimes several passages through inverted selective media may be necessary to eliminate all contaminants.

B. Selection of single zoospores.

Produce zoospores axenically as described below. First, count the zoospores by placing 50μl zoospore suspension on a slide. Add 25μl of lactophenol Trypan blue solution to the zoospores and thoroughly mix. Cover the zoospore suspension with a 22 × 22 mm coverslip. Count the spores in 10 randomly chosen microscopic fields under × 200 magnification. The number of zoospores per microscopic field can be calculated using the diameter of the microscopic field (2). Dilute the spores to 100/ml. Place 200μl of spore suspension on the surface of dilute LB or V-8 media and spread with a hockey stick (0.5 cm × 25 cm glass rod with 6 cm at one end bent at a 45° angle). **Invert the agar in the. plate.** Germinated zoospores can be seen through the bottom of the plate to verify single-zoospore colonies. Pick off selected colonies from the surface and purify as described above.

C. Selection of single oospores.

Phytophthora spp. are reputed to be both heterokaryotic and heterozygous in nature. It may be desirable prior to testing virulence or long-term storage to reduce this variability in homothallic species by several successive single oospore propagations. The following method was developed by Bhat *et al.* (1) for *P. sojae.* Place 5 ml of dilute LB extract in a 25-ml, screw-capped, autoclavable, scintillation vial. Inoculate with 4 agar culture plugs, 0.25 cm^2, from the margin of a colony growing on dilute LB agar. Incubate 30 days at room temperature on the laboratory bench, without special control of light. Transfer the culture to a 5-ml screw-cap bottle. Add 2000 units/ml of B-Glucuronidase (Type H-1, Sigma) to bring the volume to 4 ml. Place the probe of a Tissue-tearor (Biospec Products, Bartlesville, OK) into the culture suspension and macerate for 1 min at 1000 rpm. Incubate the culture at 37 C for 12 hr. Place suspension into a sterile 15-ml centrifuge tube. Add distilled water to a volume of 13 ml. Centrifuge 10 min at 3000 rpm in a lab-top centrifuge. Pour off the liquid and add 13 ml for a second wash. Centrifuge at 2000 rpm for 5 min. Repeat for a total of 4 washes. Suspend oospores in 10 ml of distilled water and freeze at −20 C. After a minimum of 24 hr, thaw at 45 C, remove mycelial debris from the surface with a pasteur pipette and centrifuge to concentrate spores. Spread 200 oospores on the surface of 1.5% water agar containing 10 μg/ml of cholesterol and rifampicin. Incubate on the laboratory bench (ca. 25 C). Generally, single-oospore cultures can be seen on the surface of the agar in 1 week.

D. Storage on agar.

Most *Phytophthora* species will survive well on a slant (9 ml of agar in a 25-ml scintillation vial) of a moderately weak media such as the extract from 40 ml V-8 juice/L of agar. Allow the cultures to grow for 1 week at room temperature. Add 2 ml of sterile distilled water. Store at 10 C in the dark. Vial caps should be tight to minimize evaporation. Generally, yearly transfers are adequate to maintain cultures. If the agar dries, the surface can be flooded with selective media cooled to 45 C at a right angle with the dried agar surface. Viable mycelium or oospores should produce new mycelium within 1 week. Species that do not produce resistant structures, such as *P. capsici*, may require more frequent transfers. Some cultures such as *P. infestans* and *P. fragariae* may require special media and temperatures for storage and need to be checked in the literature.

E. Storage in water.

Most *Phytophthora* cultures can be stored for a long time in water culture at room temperature. Either sterile distilled water or 1 hemp seed or comparable amount of plant tissue autoclaved in 5 ml distilled water are suitable. Place a 0.5 cm square or disc from the edge of a growing culture in 5 ml of the liquid, allow the culture to grow into the water for 1 week, screw the lid down tight and store at room temperature in the dark. The only disadvantage of this method is imperfectly sealed caps that permit evaporation and drying of the culture. A moisture-proof seal is necessary, while gas exchange is not.

F. Storage in liquid Nitrogen

Considerable success has been achieved in storage of *Phytophthora* spp. in liquid Nitrogen. See Tooley (19) for details.

III. Identification of *Phytophthora*

Identification of *Phytophthora* spp. is based on differences in sporangial, oospore, chlamydospore and hyphal as well as physiological characteristics. Therefore, it is useful to have simple methods for inducing formation of these structures.

A. Sporangia production.

1) *On agar:* The foliar or above-ground *Phytophthora* spp. (i.e., *P. cactorum, P. infestans* and *P. parasitica*) readily produce sporangia on solid agar media. Dilute lima bean, V-8 juice or carrot agars are suitable for most. *P. infestans* requires concentrated frozen lima bean agar. Sporangia production in *Phytophthora* is dependent on light. A good source of light for the laboratory is 40-watt, cool-white, fluorescent tubes placed about 60 cm above the cultures. Continuous illumination is most effective.

2) *In liquid culture:* The root- and crown-rotting *Phytophthora* spp. (i.e., *P. cryptogea, P. erythroseptica, P. megasperma*) produce sporangia best in liquid culture. A good method for most of these species is to take a block of agar (1.0 cm^2) from the edge of rapidly growing colonies and place it in a petri dish containing 15 ml of sterile distilled water. Incubate low temperature isolates at 18 C and others at 25 C. The best agar medium for production of mycelium for water cultures may be different for different species. Place the water culture under light as described above. Dilute extracts from hemp (1 seed/15 ml) or frozen lima beans (20 g/liter) also may be effective. Many of the species in this group also produce sporangia if young cultures are washed repetitively with sterile distilled water and placed under light. The hemp seed water culture is suitable for most species. Autoclave one hemp seed (*Cannabis sativa*) in 10 ml distilled water with 0.15% agar for 15 min. Pour the hemp seed and dilute agar into a petri dish. Split the hemp seed. Place an agar culture plug adjacent to the hemp seed halves. Incubate for 3-5 days for sporangia. This method does not work for *P. cinnamomi*. Note: Agar may be inhibitory to sporangia production. Hemp seed in water may be more effective.

3) *Salt washing:* Some *Phytophthora* spp., notably *P. cinnamomi*, do not form sporangia either on solid or in liquid media even though they are exposed to light. Originally it was found that these species produced sporangia if placed in a nonsterile soil extract. Subsequently, it was found that sporangia could be produced axenically by washing young, actively growing mycelium in specific salt solutions (6). Salt washing also is beneficial for other *Phytophthora* spp., such as *P. sojae,* if large numbers of zoospores are needed. The following salt-washing method has worked well in our laboratory: Produce young, active colonies of *Phytophthora* on dilute lima bean agar (extract from 50 g frozen lima beans/L). Place 15 pieces of culture from the edge of the colony (4 mm in dia.) into 25 ml of lima bean extract (as above). Care should be taken to transfer as little agar as possible. After 40-48 hr, pour off the culture medium and replace with 25 ml of

Chen-Zentmeyer's salt solution. Be sure that the salt solution is adjusted to pH 7.0. At hourly intervals (15 min for *P. sojae*), replace the salt solution four times. Incubate in light after the final washing. Sporangia of *P. cinnamomi* form in about 9 hr and those of *P. sojae* in about 5 hr. To obtain zoospores, replace the salt solution with sterile distilled water and chill briefly. If the final salt wash is replaced by 20 ml sterile distilled water, sporangia will form zoospores as soon as they are mature.

B. Oospore production.

1) *Homothallic species:* Some *Phytophthora* spp. readily form oospores in single strain or monospore cultures. They are considered to be homothallic and form all the hormones required for antheridia and oogonia production in single culture (12). Sterols are required for oospore production. Generally plant extract media have adequate sterols. Dilute lima bean agar and carrot agar are generally satisfactory. Media can be supplemented with sterols by adding 10 mg/l in a carrier such as dimethylformamide and thoroughly dispersing before autoclaving. Some species produce oospores abundantly, i.e., *P. cactorum* and *P. sojae.* Other homothallic species do not form oospores abundantly, i.e., *P. medica-ginis, P. syringe and P. lateralis.* In such species oospores may form after one month or more and may be found only in the inoculum piece. Light inhibits oospore formation.

(2) *Heterothallic species:* Some *Phytophthora* spp. lack or do not have receptors for both hormones required for induction of oospores. These species are called heterothallic and do not produce oospores unless paired with the complementary compatibility type, A^1 or A^2, which provides the missing hormone (12). Carrot, hemp or lima bean seed extract agars are suitable for most pairings. Cultures that are to be paired should be placed 2 cm apart on agar media in the middle of a petri dish.

C. Chlamydospores and hyphal swellings.

Some *Phytophthora* spp. (i.e, *P. cinnamomi, P. lateralis* or *P. parasitica*) have distinctive chlamydospores. Generally, they form readily in agar culture or water culture and do not require special methods. Many *Phytophthora* spp. produce hyphal swellings. Those produced by *P. cryptogea* are typical and abundant.

IV. Keys for Identification

There are two excellent keys for identification of *Phytophthora*. The one by Waterhouse (24) groups the species by sporangial characteristics. The second by Newhook *et al.* (14) uses other characteristics as well. Below is a simplified key for the common species that has been devised using characteristics used by both keys.

Artificial Dichotomous Key to Common *Phytophthora* Species

1	No growth at 25 C	14
1	Growth at 25 C	2
2	Host specific	3
2	Not host specific	4
3	On Solanaceae	*P. infestans*
3	On Phaseolus	*P. phaseoli*
3	On Fragaria	*P. fragariae*
3	On soybean	*P. sojae*
3	On Medicago or Cicer	*P. medicaginis*
4	Oospores readily formed in single culture	5
4	Oospores rare in single culture	9
5	Paragynous antheridia	6
5	Amphigynous antheridia	8
6	Papillae conspicuous, thick (4μm or more)	*P. cactorum*
6	Papillae not conspicuous	7
7	Papillae 1-4μm thick	*P. citricola*
7	Papillae less than 1μm thick	*P. megasperma*
8	Oogonia stalk tapered	*P. heveae*
8	Oogonia stalk not tapered	*P. erythroseptica*
9	Sporangia persistent (noncaducous)	10
9	Sporangia caducous	13
10	Sporangia with thick papillae (papillate) Sporangia readily formed on solid agar	11
10	Sporangia with thin papillae (nonpapillate) Sporangia rarely formed without washing agar	12
11	Sporangia round, many intercalary	*P. parasitica*
11	Sporangia ovoid—terminal	*P. citrophthora*
12	Large botryose hyphal swellings (Chlamydospores)	*P. cinnamomi*
12	Hyphal swellings smaller, not botryose	*P. cryptogea*
13	Pedicel (sporangial stalk) short	*P. palmivora*
13	Pedicel long—greater than 20 μm	*P. capsici*
14	No sporangia on agar, lateral chalmydospores	*P. lateralis*
14	Sporangia produced on agar	15
15	Papillae thick	*P. hibernalis*
15	Papillae thin	*P. syringae*

V. Simple Pathogenicity Tests

There are many types of pathogenicity tests available. The two described here are highly artificial and utilize agar cultures. They were developed to evaluate cytoplasmic resistance in plants. One involves placing parts of an agar culture in a wound and the second involves root inoculation. Both are very severe tests and should be considered only as preliminary tests.

A. Hypocotyl injection test.

This is a hypocotyl inoculation test modified from Haas *et al.* (9). Germinate test units, i.e., large-seeded legumes such as soybean, in coarse vermiculite until the cotyledons have expanded (about 6 days). To obtain good emergence with *Phomopsis*-infested or poor-quality seed, spray the seed to run-off with a solution of 0.3 g benomyl (0.6 g Benlate 50, E. I. Dupont Nemours and Co., Wilmington, DL) per liter of water. Grow inoculum in soft (12 g agar/L) dilute lima bean or V-8 juice agar until the mycelium covers the plate. Cut the culture in strips and place in a 10-ml syringe. Force the agar culture through the syringe. Reload the syringe with the macerated culture and put on a #18 needle. Make a slit about 1 cm long in the hypocotyl of the seedling just below the cotyledonary node with the needle tip. Place 0.2 to 0.4 ml (approximately 200 to 400 cfu/ml) of the culture slurry into the slit with the syringe. Cover the plants with plastic for about 12 hr to prevent the agar from drying before infection can take place. Incubate at the desired temperature in diurnal light for 1 week. Susceptible plants will die or develop distinct lesions. Resistant plants or nonhosts will develop a hypersensitive reaction. Virulence on older plants can be tested with this method, but slitting older stems with the needle tip is more difficult. Also, age-related or rate-reducing resistance may give ambiguous results in older plants. Note that this is a wound-inoculation method and may bypass some natural resistance mechanisms.

B. Inoculum layer method.

This test involves placing an agar culture below the seed at planting time, as modified from Walker *et al.* (23). Grow isolates on dilute LB or V-8 agar until the colony covers the surface. Punch 3 holes, 5 mm dia, in the bottoms of 32-oz polystyrene containers. Fill the bottom of the container with 11 cm of coarse agricultural vermiculite and wet thoroughly. Remove the agar culture from the plate intact and place on the surface of the vermiculite. Cover with 2-5 cm of coarse vermiculite and water. Place 15 seed on the surface of the second layer of vermiculite. For *Phomopsis*-infested or poor-quality seed, spray the seed to run-off with a solution of 0.3 g benomyl (0.6 g Benlate 50, I.E. Dupont Nemours, Wilmington, DL) per liter of water. Then cover the seed with 2 cm of coarse vermiculite and water again. Water the containers as needed. Seedlings should emerge within 1 week. Infected seedlings will be stunted and may die. Three weeks after planting, the roots can be removed from the container and the severity of root rot rated. The following rating system has been found useful:

1=no root rot

2=trace of root rot

3=bottom third of root mass rotted

4=bottom ⅔ of root mass rotted

5=all roots rotted, 10% seedling kill, slight stunting of tops of plants

6=50% seedling kill, moderate stunting of tops

7=75% seedling kill severe stunting of tops

8=90% seedlings killed

9=all seedlings dead

10=all seedlings killed before emergence.

Resistant interactions give a "1"- or "2"-type reaction.

VI. Summary

All of the techniques described are effective for various species of *Phytophthora*. Standard procedures used in our laboratory for handling *P. sojae* are as follows: Ethanol for surface sterilization; PBNIC medium for isolation from diseased tissues; soybean seedling baiting for isolation from soil; induced sporangia and zoospore production for seedlings or isolation from the inside of large tap roots for separating *Phytophthora* from *Pythium* in root infections; salt washing for production of zoospores; isolation of single zoospore or oospore cultures as describe above; storage at 10 C on agar; virulence evaluation with the hypocotyl inoculation method; and evaluation of aggressiveness (speed with which roots are rotted) using the inoculum layer technique. Other methods may be more useful for other *Phytophthora*-crop combinations.

VII. Media and Reagents

Chloral hydrate-acid fuchsin (7)

Chloral hydrate	830 g
Distilled Water	100 ml
Acid fuchsin	.01 g

Heat mixture in hood to dissolve. This quantity of chloral hydrate produces a saturated solution at 25 C. Trypan blue may be substituted for acid fuchsin.

Lactophenol—Trypan blue solution

Phenol (warmed to melt)	20 ml
Lactic acid	20 ml
Glycerin	40 ml
Distilled water	20 ml
Trypan blue	0.01 g

Dissolve 0.01 g of trypan blue in 100 ml of the above solution.

Chen and Zentmyer's salt solution (6)

$Ca(NO_3)_2.4H_20$	0.01 M
$MgSO_4.7H_20$	0.004 M
KNO_3	0.005 M
Sequestrene 138 (6% Fe)	0.084 mg/l

This salt mixture is for washing cultures to induce sporangia production. The solution can be autoclaved to sterilize. Fe can be added as $FeSO_4.7H_20$ after autoclaving at rate of 0.02 mg/liter. Filter $FeSO_4.7H_20$ through a 0.22-micron millipore filter to sterilize. Iron sequestrene is more convenient and the preferred source of Fe.

Lima bean agar

Frozen lima beans	150 g
Distilled Water	1000 ml

Autoclave frozen lima beans in 500 ml of water for ½ hr. Pass through a household sieve to remove seed coats. Add 20 g agar to extract and pulp and increase volume to 1000 ml. Autoclave and pour plates or dispense into tubes and autoclave.

Dilute frozen lima bean agar

Frozen lima beans	50 g
Distilled water	1000 ml
Agar	20 g

Autoclave frozen lima beans in 500 ml of water. Pass through a household sieve. Filter through a pad of diatomaceous earth (Sigma D 5509) on Whatman #1 filter paper. Increase volume to 1 liter. Add agar and autoclave to sterilize. Delete agar for lima bean extract medium used for producing mycelium used for salt washing to induce sporangia in *P. cinnamomi.*

Hemp seed extract agar

Hemp seed	50 g
Distilled water	1000 ml
Agar	20 g

Autoclave hemp seed in 500 ml water for 30 min. Pass through a household sieve to remove seed. Add 20 g agar and increase volume to 1000 ml. Autoclave to sterilize.

Dilute V-8 juice agar

V-8 juice	40.00 ml
$CaCO_3$	0.60 g
Bacto yeast extract	0.20 g
Sucrose	1.00 g
Cholesterol	0.01 g(2 ml, N, N, dimformamide sol.)
Bacto agar	20.00 g
Distilled water	1000.00 ml

Autoclave the V-8 juice, $CaCO_3$ and distilled water for 15 min at 121 C; filter through Whatman #1 filter paper with a 1-cm pad of diatomaceous earth; increase volume to 1000 ml with distilled water; add sucrose and yeast extract; add cholesterol and disperse by shaking; add agar and autoclave. A V-8 juice concentrate can be prepared by adding 0.6 g $CaCO_3$ to 40 ml V-8 juice, autoclaving, and storing frozen and unfiltered until needed. Note: There are many variations of V-8 juice agar. Variables in preparation include amount of V-8 juice (200 ml/l is considered standard), additives used and clarification (none, filtered or centrifuged).

Carrot agar

Carrots	200.0 g wet weight
Distilled water	1000 ml
Agar	20.0 g

Blend carrots fresh with 500 ml water. Squeeze the juice through 2 layers of cloth. Add agar and water to juice to bring to 1000 ml and autoclave.

Tsao-Ocana medium, PVP (22)

Corn meal agar (Difco)	17.0 g
Pimaricin	0.010 g
Vancomycin	0.200 g
PCNB	0.100 g
Distilled water	1000 ml

Add antibiotics after autoclaving. Store in dark. Incubate plates in dark since Pimaricin is inactivated in light. Hymexazol may be added to this medium at 50 mg/l for control of *Pythium* but may be inhibitory to some species of *Phytophthora* (21).

PBNIC inhibitors (15)

Benlate (50% benomyl)	0.010 g
Terrachlor (75% pentachloronitrobenzene)	0.054 g
Rovral (50% Iprodione)	0.040 g
Neomycin sulfate	0.100 g
Chloramphenicol	0.010 g

These inhibitors may be added to dilute V-8 juice agar before autoclaving. Hymexazol can be added for control of *Pythium* but is quite toxic to *P. sojae* and possibly other species (21).

Canaday-Schmitthenner inhibitors (4)

Dilute V-8 Juice medium	see above
PCNB	0.020 g
Benomyl	0.010 g
Hymexazol	0.020 g
Neomycin sulfate	0.100 g
Rifampicin	0.009 g

These inhibitors are added to dilute V-8 agar for isolating from soybean leaf disc baits. All ingredients except rifampicin can be added before autoclaving. Rifampicin is added after autoclaving in 5 ml of 95% ethanol or 4 ml of acetone. Store in the dark and use within 2-3 weeks.

PARP medium (11)

Corn meal agar	17 g/l
Pimaricin	0.010 g
Ampicillin	0.250 g
Rifampicin	0.010 g
Pentachloronitrobenzene	0.100 g

This medium can be made partially selective for *Phytophthora* in the presence of *Pythium* by addition of 20-50 mg/l hymexazol. See above for comments regarding hymexazol and storage of media with Pimaricin.

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